

Selective Labeling of Individual Neurons in Dense Cultured Networks with Nanoparticle-enhanced Photoporation

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Abstract

Neurodevelopmental and neurodegenerative disorders are characterized by subtle alterations in synaptic connectivity and perturbed neuronal network functionality. A hallmark of neuronal connectivity is the presence of dendritic spines, micron-sized protrusions of the dendritic shaft that compartmentalize single synapses to fine-tune synaptic strength. However, accurate quantification of spine density and morphology in mature neuronal networks is hampered by the lack of targeted labeling strategies. To resolve this, we have optimized a method to deliver cell-impermeable compounds into selected cells based on gold-nanoparticle mediated photoporation, termed 'Spatially resolved NANoparticle-enhanced Photoporation' (SNAP). We show that SNAP enables efficient labeling of selected individual neurons and their spines in dense cultured networks without causing cytotoxicity. We compare SNAP with widely used spine labeling techniques such as the application of lipophilic dyes and genetic sparse labeling. Using SNAP, we find a time-dependent increase in spine density in healthy cultures as well as a reduction in spine density after chemical mimicry of hypoxia. Since, the sparse labeling procedure can be automated using an intelligent acquisition scheme, SNAP holds promise for high-content screening campaigns of neuronal connectivity in the context of neurodevelopmental and neurodegenerative disorders.

Introduction

Many neurodevelopmental and neurodegenerative disorders are characterized by altered synaptic connectivity (Glausier and Lewis, 2013; Herms and Dorostkar, 2016). Today, insufficient fundamental knowledge about the underlying mechanisms at the cellular and neuronal network levels precludes the development of disease-modifying treatments.

Primary neuronal cultures are frequently used *in vitro* models in studies of neuroplasticity due to their ability to spontaneously form synaptically connected networks (Cohen et al., 2008; Verstraelen et al., 2017). Synapses, in particular postsynaptic compartments known as dendritic spines, serve as sensitive morphological correlates of neuronal connectivity since they appear, disappear or display morphological modifications in response to learning paradigms (Segal, 2017). Spine motility, and hence the extent of electrical and biochemical compartmentalization, is mediated by the actin cytoskeleton (Lei et al., 2016). With a typical length between 0.5 and 4 μm (Papa et al., 1995), spines can be resolved using high-resolution confocal microscopy. However, accurate spine quantification is complicated due to the dense nature of mature neuronal networks with many dendrites and axons crossing each other, making a sparse labeling strategy imperative. Ideally, such a method allows selecting individual neurons that are sufficiently separated or have a particular phenotype of interest. To meet these requirements, we have optimized a recently reported technique called Spatially resolved NANoparticle-enhanced Photoporation (SNAP) (Xiong et al., 2017). This approach exploits the ability of plasmonic gold nanoparticles (AuNPs) to generate vapor nanobubbles (VNBs) upon pulsed laser irradiation (Skirtach et al., 2005; Xiong et al., 2016b). When a membrane-adsorbed AuNP receives a laser pulse, it rapidly heats up, causing the surrounding water to evaporate. This results in the formation of VNBs that expand and collapse, thereby creating transient pores in the plasma membrane allowing otherwise impermeable molecules to enter the cytoplasm. Spatial selectivity, after bulk incubation with AuNPs, is achieved by directing the laser beam to the cells of interest.

In this study, we exploit the concept of SNAP for efficient delivery of the cell-impermeable and fluorescently labeled actin-binding drug phalloidin in individual primary neurons. This allows the visualization of dendritic spines with high contrast in dense neuronal networks. Using automated selection of Hoechst-labeled neuronal nuclei, we significantly increase the throughput, showing SNAP's potential for high-content screening. We assess the toxicity and selectivity of SNAP and compare with a number of commonly used techniques for sparse labeling in terms of targeting specificity, speed and ease-of-use. As proof-of-concept, we quantify maturation-dependent and hypoxia-induced alterations in spine density.

Materials and methods

Primary hippocampal cultures and hypoxia induction

This study was carried out in accordance with the recommendations of the ethical committee for animal experimentation of the University of Antwerp (approved ethical files 2014-53 and 2015-54).

Hippocampi were dissected from WT E18 C57Bl6 mouse embryos in HEPES (7 mM)-buffered Hanks Balanced Salt Solution (HBSS-HEPES), followed by trypsin digestion (0.05%; 10 min; 37°C) and mechanical dissociation. After centrifugation (5 min at 200g), the cell pellet was resuspended in Minimal Essential Medium supplemented with 10% heat-inactivated normal horse serum and 30 mM glucose. Cells were plated in Poly-D-Lysin-coated 96-well plates (Greiner Cell coat, µClear), at 45,000 cells/cm², and kept in a humidified CO₂ incubator (37°C; 5% CO₂). After 4 hours, the medium was replaced with 150 µl B27-supplemented Neurobasal medium (NB-B27), containing Sodium Pyruvate (1 mM), Glutamax (2 mM) and glucose (30 mM). To suppress proliferation of non-neuronal cells, 1 µM arabinosylcytosine was added in 50 µl NB-B27 at the third day after plating. The cultures were grown without any further medium replacement until the time of analysis. Cell culture supplies were purchased from ThermoFisher. For pharmacological induction of hypoxia, Dimethyloxallylglycine (DMOG; 10 – 100 µM; Sigma-Aldrich D3695) was added at 13 days *in vitro* (DIV) in 25 µl NB-B27, for 24h. Controls received an equal volume of sterile water in NB-B27 (final volume of 25 µl).

Spatially resolved nanoparticle-enhanced photoporation (SNAP)

At the indicated DIV, 130 µl NB-B27 was removed (~ 50 µl left) and cells were incubated at 37 °C with cationic AuNPs of 70 nm (CU11-70-P30-50, Nanopartz Inc, Zeta-potential +30 mV) in 25 µl HBSS-HEPES at a final concentration of 1.8×10⁸ particles/ml. After a 30 min incubation period, the cell-permeant nuclear label Hoechst 33342 (final concentration 10 µg/ml in 25 µl HBSS-HEPES, ThermoFisher H3570) was added to the cells and incubated for another 15 min at room temperature. Subsequent imaging and photoporation were done at room temperature while the pH was maintained by the HEPES buffer. The Hoechst-stained cell culture was imaged using a confocal laser scanning microscope (Nikon C1si, 10X NA 0.45 CFI Apochromat, λ_{ex} 405 nm, λ_{em} 440 nm). A large overview image of 1.27 by 1.27 mm was obtained using the microscope's image stitching feature. Nuclei were identified in the image as described before (De Vos et al., 2010) and a sparse selection of sufficiently separated cells was made with a custom-written Matlab script (available upon request). Afterwards, the local coordinates of the cells in the image were transformed to global coordinates of the microscope stage. Two minutes prior to the laser scanning treatment, 25 µl of AF488- or AF647-labeled phalloidin (final concentration 20 U/ml; ThermoFisher Scientific) in HBSS-HEPES was added to the cells. A homemade setup including a Nikon Ti epifluorescence microscope with programmable motorized stage and electronic timing system was used to generate and detect vapor nanobubbles (VNBs) (Xiong et al., 2014). A pulsed laser (7 ns pulses at 20 Hz) was tuned at a wavelength of 561 nm (Opolette HE 355 LD, OPOTEK Inc.) and used for illumination of AuNPs to generate VNBs. A beam expander (#GBE05-A, Thorlabs) combined with iris diaphragm (#D37SZ, Thorlabs) was used to adjust the

diameter of the laser beam to the size of a neuronal soma (~20 μm diameter). The laser pulse energy was monitored by an energy meter (J-25MB-HE&LE, Energy Max-USB/RS sensors, Coherent) synchronized with the pulsed laser. The laser fluence level was maintained at 2 J/cm^2 , *i.e.* well above the VNB threshold (~0.5 J/cm^2 @ 561 nm & 7 ns pulse duration) of the 70 nm AuNP as previously determined (Xiong et al., 2014; Xiong et al., 2016a).

Confocal imaging and image quantification

After photoporation, the sample was placed back in the incubator for at least 1 hour to allow the cells to recover and permit equal phalloidin distribution throughout the targeted neuron. After gently washing excess phalloidin in the culture medium, labeled cells were imaged with a confocal laser scanning microscope using both low (10X NA 0.45) and high (60XW NA 1.20) magnification objective lenses ($\lambda_{\text{ex}}=488$ nm, $\lambda_{\text{em}}=525\pm25$ nm or $\lambda_{\text{ex}}=647$ nm, $\lambda_{\text{em}}=700\pm40$ nm). To visualize the entire neuronal network, the membrane-permeable dye SiR-tubulin (1 μM final concentration) was added for 30 min prior to imaging ($\lambda_{\text{ex}}=647$ nm, $\lambda_{\text{em}}=705\pm45$ nm). Confocal images were acquired within 2 hours after recovery from the photoporation procedure. Quantification of dendritic spine density, *i.e.* the number of spines divided by the dendrite length, was done manually with ImageJ software (Schneider et al., 2012).

Determination of cell viability

The following approach was adopted to screen for potential cytotoxic effects of the photoporation procedure in 14 DIV cultures. First, the laser beam scanned a pre-defined square pattern covering most of the well (diagonal of the square = diameter of the well). Laser pulse frequency, laser beam diameter and stage speed were adjusted so that each location within the square received a single laser pulse. AuNP concentration, incubation times and laser fluence were identical to those used for image-guided SNAP. The following conditions were considered: -AuNP/-laser; +AuNP/-laser; -AuNP/+laser; +AuNP/+laser. Immediately after the photoporation procedure, the wells were replenished with conditioned medium and placed in an incubator at 37 $^{\circ}\text{C}$ for 2 or 24 hours. During the last hour, the cultures were incubated with 10 μl PrestoBlue reagent (ThermoFisher A13261), after which the fluorescence was measured in a plate reader ($\lambda_{\text{ex}}=535$ nm, $\lambda_{\text{em}}=590$ nm).

Alternative spine labeling strategies

Lipophilic dye labeling – 14 DIV cultures were fixed (2% paraformaldehyde in 0.1 M phosphate buffer, 20 min at RT) and stained with the carbocyanine dye CM-DiI (1 $\mu\text{g}/\text{ml}$ in PBS, 20 min, ThermoFisher C7001). At least 24 hours passed between staining and imaging, allowing the hydrophobic dye to spread throughout the plasma membrane.

Mixed culture – Hippocampi were dissected from WT and Black6.Cg-Tg(Thy1-YFP)16Jrs/J mice (the Jackson laboratory #003709) as described above. After obtaining the single cell suspension, the YFP neurons were mixed with WT hippocampal cells at a 1:5 ratio. Cultures were grown in standard conditions until live imaging (14 DIV).

Sparse transduction – At 12 DIV, primary neurons were incubated with CellLight Actin-RFP, BacMam 2.0 particles (MOI 15, ThermoFisher C10583). This transduction protocol led to the transduction of a few dozen neurons per well, with high expression levels at 14 DIV.

Photoconversion – To obtain Adeno-Associated Viral (AAV) particles encoding for an actin-binding photoconvertible protein, a mEos4b-LifeAct7 construct (Addgene #54813 deposited by Michael Davidson) was cloned into an AAV backbone with hSyn1 promoter (Addgene #51085 deposited by Jonathan Ting), followed by the generation of AAV particles using an AAV-DJ helper free packaging kit (Cell Biolabs VPK-400-DJ). Transduction at 0 DIV (0.5 μ l crude lysate/well) resulted in the expression of the green (λ_{ex} 488 nm) mEos-LifeAct variant in the entire neuronal network. At 14 DIV, live cultures were placed on a confocal spinning disk microscope (UltraVIEW VoX, PerkinElmer) with photoconversion functionality (PhotoKinesis device, PerkinElmer). Every 2 minutes, a spot-shaped photoconversion pulse (405 nm, 100% laser power, 10 ms, diameter \sim 5 μ m) was delivered in the middle of a neuronal cell body, after which images were acquired in the green (non-photoconverted; λ_{ex} 488 nm, λ_{em} 525 nm) and red channel (photoconverted; λ_{ex} 561 nm, λ_{em} 615 nm).

Experimental design and statistical analysis

For determination of cell viability with PrestoBlue, 1 isolation was considered with 6 wells per condition. For quantification of dendritic spine density, three independent isolations were considered. For each isolation, $>1000 \mu$ m of dendrite stretches were analyzed on 10 selected neurons for each DIV or DMOG concentration. Statistical analyses were carried out in SAS JMP Pro 12 software, and the detailed results of those tests are reported in Table 1. Shapiro-Wilk tests were used to check for normality. Since some of the data sets were not normally distributed, non-parametric tests were performed throughout the paper. To assess the overall effect, Mann-Whitney (2 groups) or Kruskal-Wallis rank sums (>2 groups) tests were performed. When assessing the influence of culture age or DMOG treatment on spine density, these tests were performed within the method group (SNAP or DiI). For PrestoBlue measurements, these tests were performed within the time group (2 or 24 hours). Conditional to the overall Kruskal-Wallis test, post-hoc tests were performed. To detect differences across DIVs, Dunn all pairs tests for joint ranks were used as the non-parametric alternative for Bonferroni tests (Dunn, 1964). For the DMOG experiment, a Steel test for comparison with control was used as the non-parametric alternative for a Dunnett test (Steel, 1959). The data are represented as bar charts (mean + standard deviation).

Results

SNAP enables fast and targeted labeling of primary hippocampal neurons and their spines

Since dendritic spines contain high actin concentrations, fluorescently labeled phalloidin, a well-known cell impermeable F-actin stain, was used to label spines (Fig. 1a). SNAP can be performed in whole-field mode by visiting adjacent tiles with the microscope stage, yet a considerable advantage over current labeling techniques is the possibility of targeted labeling. Neurons can be selected based on the transmission image, or on a fluorescent label. To quickly and automatically label a sparse set of neuronal cells within the dense network, we made use of automated, image-guided SNAP (Fig. 1b, c). First, hippocampal cells were labeled with the membrane-permeable stain Hoechst. The positions of individual nuclei were identified by image processing and a random selection was made under the constraint that selected cells should be at least 200 μm apart (Fig. 1c, gold circles and arrowheads). The coordinates of the selected cells were subsequently used for SNAP-assisted phalloidin labeling. Each cell received one 7 nanosecond laser pulse at the indicated position with a beam diameter of $\sim 20 \mu\text{m}$. As demonstrated in Fig. 1c, only the targeted cells were successfully labeled. Since the photoporation beam was centered onto the nuclear area, phalloidin initially entered the soma of the cell, after which it diffused throughout the cell into the dendrites and labeled the dendritic spines (Fig. 1a). Although there is a possibility that non-targeted neurons whose neurites were in close proximity to the targeted soma may become transiently permeabilized as well, we did not observe aspecific labeling in non-targeted neurons, with the acquisition settings that were sufficient for imaging the targeted neuron. Therefore, possible aspecific labeling did not impede the selective visualization of the targeted neuron. The inset in Fig. 1c shows that dendritic spines (arrows) could be unambiguously resolved, thus enabling quantitative morphological analysis.

SNAP-assisted phalloidin labeling is selective and non-cytotoxic

Even in very dense cultures that were grown for 18 DIV, SNAP maintained target specificity allowing sparse labeling (Fig. 2a). Furthermore, SNAP could be used to sequentially label individual neurons in the same field of view in different colors (Fig. 2b). The same repetitive SNAP procedure could also be used to label different cell types in a single culture, such as neurons and astrocytes (Fig. 2c). To evaluate potential cytotoxicity induced by the SNAP procedure, cell viability was determined with PrestoBlue-based spectrophotometry after whole-field SNAP (Fig. 2d). Neither incubation with AuNPs, nor illumination with the pulsed 561 nm laser elicited a cytotoxic effect after 2 or 24 hours. Moreover, the combination of AuNPs and laser irradiation, and hence the formation of VNBs, did not decrease the viability of the cells, as was also shown by the microscopic image of a neuron that was labeled 24 hours before (Fig. 2e).

SNAP outperforms alternative labeling strategies

To expose the advantages of SNAP, we compared it with existing spine labeling strategies. The current standard for spine labeling is the use of the lipophilic dye DiI for membrane staining (Fig. 3a). Although the intense labeling allowed spine identification in selected regions, the images needed to be manually scrutinized to find the dendrite

stretches that could be unambiguously analyzed. In a randomly acquired image dataset, less than 5% ($3.2 \pm 2.8\%$) of the actual stained surface area was amenable to spine analysis (Suppl. Fig. 1a). Only a low efficiency was reached because DiI labeling is inherently stochastic and was associated with a number of artefacts, such as debris, membrane fragments and uneven fluorescence intensity, as well as labeling of non-neuronal cells (e.g., astrocytes). An alternative method that we tested to label spines in dense cultures is based on a genetically encoded fusion protein actin-RFP (BacMam actin-RFP). We tuned the transduction protocol so as to obtain low efficiency, yielding only a few dozen labeled neurons in the network with high expression levels (Fig. 3b). Dendritic spines could clearly be resolved, but again, lack of spatial control resulted in labeling of adjacent neurons (clusters) and variability in labeling efficiency across replicates. Moreover, spine head diameters were increased compared to other labeling protocols (Suppl. Fig. 1b), pointing to overexpression artefacts. To avoid these enlarged spine heads, we tested a third approach by mixing a culture of genetically labeled cells with non-labeled cells. This was achieved by using Thy1-YFP mouse-derived neurons, which constitutively produce YFP throughout the cell body. When mixing these neurons with neurons from a WT Black6 mouse at a 1:5 ratio, sufficiently sparse cultures were obtained to resolve individual spines (Fig. 3c). Although these cells integrated both morphologically and functionally in the network (Suppl. Fig. 2a-c), the rather limited YFP intensity in spines and the stochastic, sometimes clustered, distribution of YFP neurons in the well, render this method suboptimal for automated spine analysis. Furthermore, in aged (21 DIV) cultures we found that the YFP signal accumulated in all neurons ($>>1:5$). This again increased the background signal and hampered spine identification, especially at later time points (Suppl. Fig. 2d). A crucial drawback of the aforementioned strategies remains the lack of spatiotemporal control. Therefore, we also explored the potential of a targeted approach based on photoconversion of an actin-binding fluorescent protein (Fig. 3d). AAV-mediated expression of mEos-LifeAct resulted in green fluorescent signal in all neurons of the network. 405 nm photoconversion pulses were directed to the soma of a selected neuron, eventually leading to a bright red fluorescent signal in proximal as well as distal neurites of the targeted neuron. However, repeated photoconversion pulses (1 pulse every 2 minutes for 8 hours) were needed to convert a sufficient amount of actin binding proteins to fill up the very extremities of the neuron. Thus, next to the fact that this is a slow procedure, it puts a high energy load onto the cell which increases the risk on inducing phototoxicity. In contrast, the SNAP procedure requires a single nanosecond laser pulse per cell to permeabilize its membrane, allowing hundreds of cells to be labeled in one run, followed by a 1h incubation period to allow intracellular diffusion of the phalloidin (Fig. 1a). Owing to its' high selectivity, SNAP raises the labeling efficiency of analyzable dendrite stretches to 25% ($24.6 \pm 9.4\%$) as compared to the gold standard (DiI labeling) (Suppl. Fig. 1a), whilst retaining the morphological integrity of spines (Suppl. Fig. 1b).

SNAP reveals maturation-dependent and hypoxia-induced changes in spine density

Spine density is often used as a quantitative readout for synaptic connectivity within the neuronal network (Papa et al., 1995; Verstraelen et al., 2014). To further validate the SNAP approach, we compared its performance with the standard, but stochastic and artefact-prone lipophilic dye (DiI) labeling. The high-resolution images of Fig. 4a show SNAP phalloidin-labeled neurons at 7, 14 and 21 DIV, in which spines could clearly be resolved. After quantification, an age-dependent increase in spine density was detected

(Fig. 4b), with a strong commonality between SNAP and DiI labeling (Pearson's correlation 0.67). DMOG, an inhibitor of prolyl-4-hydroxylase and inducer of hypoxia-inducible factor, has previously been shown to induce spine reduction (Segura et al., 2016). Fig. 4c shows phalloidin-labeled neurons in the presence and absence of DMOG (24h, 14 DIV). Spine density was quantified on SNAP- as well as DiI-labeled cultures, treated with 0, 10 or 100 μ M DMOG for 24h (Fig. 4d). In both cases, our quantifications revealed a significantly reduced spine density after DMOG treatment.

Discussion

With our work, we have demonstrated the potential of a novel intracellular delivery method based on AuNP-sensitized photoporation to selectively label primary neurons and their spines in dense neuronal networks. SNAP offers a level of spatiotemporal control that is not available with commonly used stochastic labeling methods, and it is less time- or energy-consuming compared to alternative targeted methods such as photoconversion. To visualize dendritic spines, the fluorescently functionalized actin-binding dye phalloidin was introduced in living neurons. This was done in a spatially resolved manner, after intelligent selection of neurons that were sparsely distributed throughout the network. As a proof-of-concept, maturation-dependent spine density was investigated, which increased over time in line with previous reports (Papa et al., 1995). Chemical induction of hypoxic stress led to a decreased spine density, in accordance with a study where spines were visualized by overexpression of cytosolic fluorescent proteins (Segura et al., 2016). For the current study, which focuses on spine labeling rather than analysis, only spine density and not morphology was quantified since the latter would require super-resolution imaging. Furthermore, the exact link between spine shape and synapse strength remains to be elucidated (Segal, 2017).

SNAP was shown to be non-toxic, which can be attributed to the fact that a single visible light pulse (laser fluence: 2 J/cm²) is used, and that the conversion of light into mechanical energy by the AuNPs is highly efficient and remains local (Xiong et al., 2016b). Although we have successfully acquired images of neurons that were labeled 24 hours earlier, it must be noted that phalloidin stabilizes actin filaments (Cooper, 1987) and may therefore alter the properties of the spines. Given its inability to cross the plasma membrane, phalloidin was rarely used in living cells. Yet, one report documents alterations in the growth and migration of epithelial cells and fibroblasts after micro-injection of phalloidin (Wehland et al., 1977). However, these authors have used high phalloidin concentrations (0.2 and 1 mM in the microinjection solution) compared to our study (0.7 μ M in the extracellular medium). Although the latter may explain why we managed to image a healthy-looking neuron after 24 hours, it is advisable to use other, more inert dyes such as fluorescently-labeled dextrans for long-term imaging.

Even though it was shown before that a precisely focused beam of highly intense light could permeabilize the plasma membrane with subcellular resolution (Tirlapur and Konig, 2002; Barrett et al., 2006), the use of sensitizing AuNPs allow reducing laser energy and irradiate the entire neuronal soma at once, thereby significantly increasing throughput. Upon AuNP sensitization, the laser energy per cell can typically be reduced about 15 times (10 vs 150 μ J per cell), based on comparison of published reports (Xiong et al., 2014; Georg Breunig et al., 2015).

Table 2 lists a number of potential strategies for sparse spine labeling with their targeting capability, speed, spine signal-to-background ratio, ease-of-use and drawbacks. The current gold standard for spine labeling consists in applying hydrophobic dyes such as DiI via bath application or diolistic labeling (Cheng et al., 2014). The latter makes use of a ‘gene gun’ to shoot DiI-coated bullets into thick (*e.g.*, 150 μ m) tissue slices, but is less commonly used for labeling of 2D cultures (Staffend and Meisel, 2011). DiI labeling is associated with several staining artefacts, such as uneven dye loading, clustered cells and lack of cell type specificity, that preclude automated image analysis. Genetic labeling is a frequently used alternative approach, but overexpression of fusion proteins always comes with the concern of inducing artefacts, as also we noticed by the increased spine head size after actin-RFP overexpression. This artefact was not observed after mEos-

LifeAct expression, since the fluorescent protein (mEos) was targeted to endogenous actin via the LifeAct sequence (Riedl et al., 2008), rather than expressing additional actin on top of the endogenous levels. Also, in Thy1-YFP neurons, spine heads were not enlarged, since the fluorescent protein does not specifically accumulate in spines but moves freely in the cytoplasm. However, the background YFP signal increased markedly over time, making reliable spine analysis difficult. Since such increase in background fluorescence was not seen in normal, *i.e.*, non-transgenic, non-mixed cultures, it may be possible that cytosolic YFP was transferred to non-transgenic neurons in the mixed culture. However, to our knowledge, inter-neuronal YFP transfer was not yet documented in literature. A detailed analysis of the presumed transfer fell outside the scope of our study, and would require quantification of the fraction of YFP+ neurons in mixed cultures with a neuronal counterstain, as well as microfluidic separation of YFP+ and non-transgenic neurons. Although not documented for YFP, inter-neuronal protein transfer was already studied in detail for α -synuclein (Desplats et al., 2009), β -amyloid (Song et al., 2014) and Tau (Wang et al., 2017). Potential mechanisms that were shown to be involved include synaptic transfer (Dujardin et al., 2014), exosomal exchange (Chivet et al., 2014) and transport through tunneling nanotubes (Tardivel et al., 2016).

In non-targeted labeling approaches, the stochastic and sometimes clustered distribution of cells throughout the well complicates automated spine analysis. Targeted labeling strategies are, therefore, highly desirable. Micro-injection in selected cells has been successfully used, but this approach lacks the speed of the SNAP method (Dumitriu et al., 2011; Chow et al., 2016). As an alternative for SNAP, we explored the potential of an actin-binding photoconvertible protein, but found that repeated 405 nm laser pulses were needed to reach sufficiently high levels of the photoconverted variant. In case of SNAP, a single light pulse was sufficient to label a selected neuron. Therefore, the throughput for labeling hundreds of neurons was significantly higher compared to the photoconversion approach. As such, SNAP represents a non-toxic approach for spine labeling that is superior to commonly used protocols in terms of speed and selectivity.

Though the current study involves the selective labeling of actin and hence dendritic spines with phalloidin, SNAP's range of potential applications is much wider. First, the selective labeling can be directed to specific neurons that show particular geno- or phenotypic patterns of interest. These may be other cell types such as astrocytes, neuronal subtypes (*e.g.* pyramidal- or interneurons) or neurons that show intracellular accumulation of toxic proteins (*e.g.* α -Synuclein or Tau). Second, consecutive labeling rounds with different fluorochromes allows studying the connectivity pattern between neurons of different subpopulations. Proof for the feasibility of both of these applications was provided in this study. Third, in addition to labeling selected cells with impermeable fluorescent dyes, it is equally possible to selectively deliver other nanomaterials such as drugs, plasmid DNA or siRNA, as previously shown in cell lines (Lukianova-Hleb et al., 2012; Xiong et al., 2014). It is of note that DNA transfection in individual neurons has been attempted before with standard photoporation (*i.e.* with a focused femtosecond laser beam without sensitizing nanoparticles). However, the success rate was very low with less than 15% of the cells being successfully transfected, and the photoporation was rather slow at ~1.7 cells/s (Antkowiak et al., 2013). **Although not yet reported in literature**, it can be anticipated that both the efficiency and speed of this procedure will increase when using sensitizing AuNPs. Lastly, SNAP bears the potential to label cells in a 3D environment such as organotypic brain cultures. However, it remains to be determined

whether AuNPs penetrate well in brain tissue, and precise axial focusing of the photoporation beam will be needed to achieve single cell selectivity.

In conclusion, the flexibility and speed with which individual cells can be labeled make SNAP an ideal tool for high-content applications, not only for selective labeling but also for targeted drug or nucleic acid delivery. This may eventually lead to a better understanding of the pathogenic mechanisms that underlie neurodevelopmental and neurodegenerative disorders.

Conflict of interest

The authors declare no competing financial interests

Author contributions

RX, PV, KB, WDV conceived and designed the experiments. RX and PV performed the experiments. RX, PV and WDV analyzed the data. RX, PV, KB and WDV drafted the manuscript. All authors critically revised the manuscript and are accountable for all aspects of the work.

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Table and figure legends

Table 1 – Detailed statistics for the executed experiments

Fig	description	condition	Shapiro-Wilk	D F	Overall test	post-hoc test
2c	Cytotoxicity	2h 24h	P=0.7953 p=0.8160	3	KW p=0.2064 KW p=0.3755	NA NA
4b	DIVs	SNAP DiI	p<0.0001 p=0.3802	2 2	KW p<0.0001 KW p=0.002	Dunn all pairs - 7-14 DIV p<0.0001; 14-21 DIV p=0.3657; 7-21 DIV p<0.0001 Dunn all pairs - 7-14 DIV p=0.0324; 14-21 DIV p=0.6017; 7-21 DIV p=0.0024
4d	DMOG	SNAP DiI	p=0.0025 p=0.0003	2 2	KW p=0.0066 KW p=0.0043	Steel with control - 10 μ M p=0.0153; 100 μ M p=0.0157 Steel with control - 10 μ M p=0.0194; 100 μ M p=0.0048
S1a	Specificity		P=0.2952	1	MW p=0.009	NA
S1b	Spine morphology		p=0.4426	4	KW p<0.0001	Dunn all pairs - mEosLA-DiI p=1; SNAP-DiI p=1; SNAP- mEosLA p=1; YFP-DiI p=1; YFP-SNAP p=1; YFP-mEosLA p=0.6875; mEosLA-BacMam p=0.0454; SNAP-BacMam p=0.0071; DiI- BacMam p=0.0064; YFP- BacMam p<0.0001
S2b	YFP spines		p=0.3817	1	MW p=0.7494	NA
S2c	Functional integration	7 DIV 14 DIV	p=0.0187 p=0.1228	1 1	MW p=0.9223 MW p=0.5485	NA NA

For all quantitative data, the p-values of Shapiro-Wilk tests for normality are listed, as are the degrees of freedom (DF) and p-values of the overall tests (KW: Kruskal-Wallis; MW: Mann-Whitney), and the nature and p-values of the post-hoc tests.

Table 2 – Comparison of sparse spine labeling strategies

Sparse labeling approach	Targeting	Speed	Spine SBR	Ease-of-use	Artefacts and disadvantages
Lipophilic dye (DiI)	-	+	±	+	Debris, uneven dye loading, clusters, no cell type specificity
Transfection (actin-RFP)	-	-	+	+	Increased spine head size, clusters
Mixed culture (YFP)	-	-	-	-	Clusters, low flexibility
Photoconversion (mEos-LifeAct)	+	-	+	-	Repeated pulses needed, risk of photodamage
SNAP (AF488-phalloidin)	+	+	+	±	Actin stabilization by phalloidin, pulsed laser needed

Commonly used spine labeling strategies are compared with SNAP in terms of targeting potential, labeling speed, spine SBR (average spine signal to local background intensity ratio), applicability and risk for artefacts.

Figure 1 – SNAP enables fast and targeted labeling of primary hippocampal neurons and their spines

a. General workflow of SNAP. Intracellular delivery of phalloidin relies on the adsorption of cell-interactive plasmonic gold nanoparticles (AuNP) to the plasma membrane. Upon selective illumination of the cell of interest with 561 nm laser light, AuNPs heat up and induce vapor nanobubbles (VNBs). These VNBs transiently open the membrane, allowing the otherwise impermeable dye to enter the soma (white arrows). After washing and allowing time for intracellular diffusion of the dye to the neuronal extremities, completely labeled neurons can be monitored with high contrast **b.** Workflow of image-guided SNAP. Before the actual SNAP procedure is initiated, large field-of-view images are acquired of cells labeled with a cell-permeable marker. After image analysis and selection, the coordinates of the cells of interest are determined and fed into the SNAP setup for targeted photoporation. **c.** Image-guided SNAP on primary neurons. Nuclei are stained with the membrane-permeable dye Hoechst. Using image analysis, all nuclei are first detected, followed by a selection of nuclei that are at least 200 μm apart (gold circles and arrowheads). The coordinates of the neurons of interest are used to guide the SNAP procedure. The high-resolution image of the phalloidin-labeled neuron shows dendritic spines (arrows) in high contrast without interference of fluorescence from overlapping dendrites or axons of nearby cells.

Figure 2 - SNAP-assisted phalloidin labeling is selective and non-cytotoxic

a. Microscopic image of an 18 DIV neuron that was selectively labeled with image-guided SNAP. The complete network is labeled with the membrane-permeable dye SiR-tubulin, whereas only the targeted neuron is labeled with phalloidin. **b.** Repetitive SNAP procedure with two chromatic phalloidin variants (AF488, AF647) allows multicolor labeling of individual neurons in the same field of view. **c.** The same procedure was used to selectively label astrocytes and neurons in different colors in the same culture. **d.** Quantification of cell viability after 2 and 24 hours shows no significant toxicity induced by the AuNPs or the 561 laser pulses, nor of the combination of both. **e.** Microscopic image of a neuron, 24 hours after SNAP-assisted phalloidin labeling.

Figure 3 - SNAP outperforms alternative labeling strategies

a. The lipophilic dye DiI enables sparse, but stochastic labeling of neurons within the neuronal network. It suffers from drawbacks such as clustered staining of adjacent neurons, debris, and uneven dye loading or overloading. **b.** Microscopic image of a neuron transduced with actin-RFP (BacMam vector). Spines can clearly be resolved since actin is highly enriched in spines. However, the overexpression of actin-RFP may lead to artefacts such as enlarged spine heads (see also Suppl. Fig. 1b) or increased cellular stress. **c.** Microscopic image of a Thy1-YFP neuron in a mixed culture with non-labeled WT cells (1:5 ratio). Spines can be discerned, but the signal of YFP in spines is weak and the labeled cell distribution is stochastic. **d.** Photoconversion of mEos-LifeAct (cyan) in a primary neuron. Every 2 minutes, a 405nm photoconversion pulse is delivered to the soma of the selected neuron, leading to a gradual spreading of the photoconverted (red) variant throughout the cytoplasm.

Figure 4 – SNAP reveals maturation-dependent and hypoxia-induced changes in spine density

a. Microscopic images of phalloidin-labeled neurons at 7, 14 and 21 DIV. Spines (arrows) can be discerned at different time points. **b.** Quantification shows a time-dependent increase in spine density and a similar trend for SNAP and DiI labeling (* $p < 0.05$ in Dunn all pairs test, see Table 1 for p-values). **c.** Phalloidin-labeled neurons with and without DMOG treatment to mimic hypoxic conditions. **d.** Quantification of spine density after SNAP or DiI labeling shows a reduction in spine density upon 24h DMOG treatment (* $p < 0.05$ in Steel test, see Table 1 for p-values). Microscopy images have been inverted for clarity.